



Faculty of Resource Science and Technology

**NITROGEN SUPPLEMENTATION WITH SAGO 'HAMPAS'
HYDROLYSATE FOR LOW COST BIOETHANOL
FERMENTATION MEDIA**

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**Nitrogen Supplementation with Sago 'Hampas' Hydrolysate for Low Cost
Bioethanol Fermentation Media**

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A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of
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DECLARATION

I hereby declare that this Final Year Project entitled “Nitrogen Supplementation with Sago ‘Hampas’ Hydrolysate for Low Cost Bioethanol Fermentation Media” is based on my original work except for the quotations and citations which have been dully acknowledged also, declare that is has not been or concurrently submitted for any other degree at UNIMAS or other institution of higher learning.



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LIST OF ABBREVIATIONS

SHH	Sago 'hampas' hydrolysate
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
HPLC	High Performance Liquid Chromatography
g/L	Gram per liter
ATP	Adenosine triphosphate
NADH	Nicotinamide adenine dinucleotide (reduced)
w/v	Weight per volume

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Nitrogen Supplementation with Sago 'Hampas' Hydrolysate for Low Cost Ethanol Fermentation Media

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ABSTRACT

Nitrogen source is very important in the production of the bioethanol. Starchy materials such as sago 'hampas' which can be found abundantly in Malaysia is one of the suitable sources for the production of bioethanol. The main focus of this study is to find out which type of nitrogen sources is the best for the fermentation of bioethanol. Previous studies showed that the best nitrogen source is the yeast extract. However, yeast extracts are expensive and this research also focus on to find which nitrogen sources are cheaper to replace yeast extract and also may decrease the production cost. Four types of nitrogen sources were used namely peptone, ammonium sulphate, yeast extract and urea. The effect of these different sources of nitrogen in the production of bioethanol were evaluated in this study. Sago 'hampas' was treated with the Liquozyme and Spirizyme for the saccharification of starch and undergo three cycles of enzymatic hydrolysis in order to get sago 'hampas' hydrolysate (SHH). Two replicates were prepared for each type of nitrogen sources. The SHH were fermented by yeast or *Saccharomyces cerevisiae* in 250 mL of Erlenmeyer flask with 100 mL of media and batch fermentation will be used in this study. From this study SHH were able to provide glucose needed during fermentation in the production of bioethanol. Urea, peptone and ammonium sulfate can replaced the yeast extract as the sources of nitrogen in fermentation.

Key words: Bioethanol, nitrogen, sago 'hampas' hydrolysate, glucose, *Saccharomyces cerevisiae*.

ABSTRAK

Sumber nitrogen adalah sangat penting dalam penghasilan bioetanol. Bahan-bahan yang kaya dengan kanji dan boleh didapati di Malaysia adalah sumber yang sesuai untuk menghasilkan bioetanol. Fokus utama dalam kajian ini adalah untuk mengenalpasti jenis-jenis sumber nitrogen yang terbaik untuk menghasilkan bioetanol. Kajian sebelum ini menunjukkan ekstrak yis adalah sumber nitrogen yang terbaik. Walaubagaimanapun, ekstrak yis adalah mahal harganya dan fokus kajian ini juga adalah untuk mengenalpasti jenis-jenis sumber nitrogen yang sesuai untuk menggantikan ekstrak yis selain untuk mengurangkan kos perbelanjaan. Empat jenis sumber nitrogen telah digunakan iaitu pepton, amonium sulfat, ekstrak yis dan urea. Penghasilan bioetanol menggunakan keempat-empat jenis sumber nitrogen telah dinilai dalam kajian ini. Hampas sago telah dirawat dengan Liquozyme dan Spirizyme semasa proses sakarifikasi dan telah menjalani tiga pusingan untuk mendapatkan hidrolisat hampas sago. Duplikasi telah disediakan untuk setiap jenis sumber nitrogen. Hidrolisat hampas sago telah menjalani proses fermentasi menggunakan yis atau *Saccharomyces cerevisiae* di dalam 250 mL kelalang kon dengan isipadu 100 mL kerja dan telah dijalankan dalam proses fermentasi kelompok. Kajian ini telah menunjukkan hidrolisat hampas sago mampu menghasilkan glukosa untuk fermentasi bagi mendapatkan bioetanol. Urea, pepton dan amonium sulfat mampu menggantikan ekstrak yis sebagai sumber nitrogen untuk proses fermentasi.

Kata kunci: Bioetanol, nitrogen, hidrolisat sago hampas, glukosa, *Saccharomyces cerevisiae*.

CHAPTER I

INTRODUCTION

Fossil fuels are currently primary source of energy. However, fossil fuels are non-renewable energy, becoming more expensive and getting lesser over the years. Nowadays, scientists are trying to find another new source of energy such as bioethanol. Bioethanol is already used as a supplement to gasoline which is about 90% gasoline is mixed with 10% of ethanol (Tortora et al., 2010). Few countries have started to use bioethanol as an alternative fuels such as Brazil, USA, China, India and Pakistan (Rubio-Arroyo et al., 2005). In Brazil, most of the sources of bioethanol are from sugar cane and in the United States a few vehicles are able to use E85 which are made up from 15% of gasoline and 85% ethanol (Tortora et al., 2010).

Lin and Tanaka (2006) mentioned that most agricultural waste containing starch can act as substrate for the ethanol fermentation by microbial processes. In Malaysia, sago palm is one of the plants that can provide high amount of starch and a sago mill can produced 25 tons of dried sago starch per day (Bujang, 2008). Therefore, a lot of sago wastes are produce and this may lead to environmental pollution. In addition, glucose is found in the sago 'hampas' which is needed for ethanol fermentation and by using sago 'hampas' can reduce the environment pollution.

Nitrogenous compounds play an important role during fermentation. According to Sridee et al. (2011), nitrogen sources are really important in producing bioethanol and brewing or winery and the sources can be from yeast extract, peptone, ammonium and urea. These nitrogen sources are able to increase yeast growth, rate of sugar utilization and reducing

the amount of fermentation (Sridee et al., 2011). In addition, Sridee et al. (2011) believed that yeast extract and peptone are better nitrogen supplements than ammonium and urea.

1.1 Problem statement

There are many factors that can affect the rate production of the bioethanol such as temperature, pH, fermentation time, agitation rate, sugar concentration and inoculum size. Therefore, research needed to be done regarding the nitrogen sources during the production of the bioethanol. For example the suitable type of nitrogen source and the amount of needed to produce high amount of bioethanol. Lack of nitrogen will cause yeast to produce hydrogen sulphide and the yeast will be not able to grow efficiently. Previous studies showed that the addition of nitrogen will increase the products of fermentation. Thus, agriculture waste might contain low amount of nitrogen which is needed for the growth of yeast during fermentation.

1.2 Objectives

The aims of this study are:

1. To identify the most suitable nitrogen source for the production of bioethanol from sago 'hampas' by *Saccharomyces cerevisiae* (*S. cerevisiae*).
2. To compare fermentation profiles of bioethanol production from hydrolysate of sago 'hampas' and commercial glucose by *S. cerevisiae*.
3. To identify which types of nitrogen source are suitable to replace yeast extract

CHAPTER II

LITERATURE REVIEW

2.1 Sago 'Hampas'

There are three major types of by-products namely bark of sago trunk, wastewater and fibrous pith residue also known as 'hampas' (Awg-Adeni et al., 2009). Sago hampas is generated during the process of starch extraction and it made up from 66% starch and 14% fibre on a dry weight basis and about 25% are made from lignin. According to Awang-Adeni et al. (2012), starch granules can be found in the untreated sago 'hampas' when observed under electron microscope. Therefore, there is a possibility to extract starch from the sago 'hampas'.

2.1.1 Sago 'Hampas' Hydrolysate (SHH)

Sago 'hampas' that had undergone saccharification of starch produced SHH. During saccharification, glucoamylase is added after gelatinization to aid the saccharification of starch (Awg-Adeni et al., 2012). Three cycles of enzymatic hydrolysis can be used to increase the concentration of the glucose. The first and the second cycle are the same procedure of enzymatic hydrolysis while the third cycle the hydrolysate is centrifuged once (Awg-Adeni et al., 2012). Awg-Adeni et al. (2012) also mentioned that 84.75 g/L of glucose produced 40.30 g/L of ethanol during the 16 hours of fermentation which means the conversion yield of total glucose in the media is 93.29%.

Table 1: Concentration of glucose produced during the cycles

Enzymatic Hydrolysis	Glucose (g/L)
Cycle I	27.79
Cycle II	73.00
Cycle III	138.45

Source from Awang-Adeni et al. (2012)

2.2 Bioethanol production from hydrolysate of agricultural waste or lignocellulosic compound

Bioethanol is produced through the fermentation of carbohydrates by the yeast. According to Lin and Tanaka (2006), biofuel is one of the most clean liquid fuels compared to fossil fuels. Therefore, the emission of carbon dioxide, nitrogen oxides and hydrocarbons are reduced. Conversion of biomass into fuel ethanol also helps the management of the agro-industrial waste products (Lin & Tanaka, 2006). Azmi et al. (2009) mentioned that starchy materials are needed to produce bioethanol. According to Lin and Tanaka (2006), there are four categories of biomass resources namely wood residues, municipal solid waste, agriculture residues and dedicated energy crops. Three main types of raw materials can be used to produce bioethanol. Firstly, sugars from sugarcane, sugar beets, molasses and fruits which these sugars can be converted directly into ethanol. Secondly, cellulose from wood, agriculture residues, wastes sulphite liquor from pulp and paper mills. Generally, mineral acids are needed to convert cellulose to sugars. Thirdly, starches from corn, cassava, potatoes and root crops which need the action of enzyme to hydrolyzed the fermentable sugars (Lin & Tanaka, 2006). Lin and Tanaka (2006) also mentioned that industrial ethanol production using many types of starchy materials such as corn, wheat, starch, potatoes, cassava root and the cheapest raw material is cassava root. Meanwhile, Zabed et al. (2014) said that sugarcane, sugar beet, sweet sorghum and others are the best

source of sugar. Direct fermentable juice can save more money than lignocellulosic materials because the sugars can directly converted into ethanol. However, cellulose materials are the best source of biomass which is mostly unutilized. 90% lignocellulose is produced and approximately 200x10⁹ tons per year (Lin & Tanaka, 2006).

Table 2: Types of agriculture waste to produce bioethanol

Agriculture waste	Ethanol production	Types of fermentation	References
Cassava (119 g)	6.15% v/v	Continuous fermentation	Rubio-Arroyo et al., 2011
Sorghum (140 g)	10.98% v/v	Continuous fermentation	Rubio-Arroyo et al., 2011
Corn stover (5 g)	12.3 g/L	Batch fermentation	Wang et al., 2012
Sugar bagasse (3 g)	0.40 g/L	Batch fermentation	Dawson and Boopathy, 2008

2.3 Nitrogen

Nitrogen is needed by the yeast during fermentation. Inadequate of nitrogen will caused the yeast to stress out and produced hydrogen sulphide (H₂S) which immediately turns to mercaptans (*Adding Nitrogen to Fermentation*, 2014). Nitrogen provides nutrients for growth and metabolic activity of the yeast during fermentation. Nitrogenous-compounds that are used by the yeast during fermentation are for biosynthetic process which are taken up and used directly, some are converted into other form and some are degraded to release nitrogen and then used by the cell (Dharmadhikari, 2001). Some research did shows that yeast extract and peptone shows more positive results than urea and ammonium sulphate (Sridee et al., 2011). Yeast extract mostly containing peptides, amino acids and vitamins and those components are very helpful in the fermentation because amino acids and

peptides are important building blocks of the cell. Therefore, this makes the yeast extract is the best nitrogen sources other than urea, peptone and ammonium sulfate. Peptone also is a natural sources amino acids and protein which can be get from enzymatic digestion or acid hydrolysis of natural products namely animal tissues, milk or plants (*Protein Sources*, 2015).

Table 3: Types of nitrogen used and the production of ethanol

Nitrogen source (g/L)	Carbon source (g/L)	Concentration of ethanol produced (g/L)	References
Peptone (2)	Glucose (50-200)	5.1-91.8	Lin and Tanaka, 2006
Ammonium sulphate (4)	Glucose (50-200)	5.1-91.8	Lin and Tanaka, 2006
Urea (6.4)	Glucose (31.6)	13.7 (max)	Lin and Tanaka, 2006
Yeast extract (9)	Glucose (30)	120.58	Wang et al., 2012

According to Ananda et al. (2011), yeast extract indeed is the best source of nitrogen source and contributes 38% of the total production cost. This is because of the shortage of the raw material resulting 10% increase of the yeast extract price. Recently, the price of the yeast extract (Bacto Difco) is about 97.58 USD (RM 712.03) per 500 g (Voigt Global Distribution, 2015), peptone (Bacto) is about 110.07 USD (RM396.85) per 500 g (SciMart, 2015), ammonium sulfate is 5.39 GBP (RM 30.27) per 500 g (Intralabs, 2015) and urea is 16. 95 USD (RM61.11) per 500 g (The Science Company, 2015). There are other nitrogen sources that can be used such as urea in carotenoid fermentation, rice bran, corn steep liquor and fish soluble waste in fermentation of ethanol (Ananda et al., 2011).

2.4 Yeast

'Sago hampas' hydrolysate (SHH) is a fermentable compound which can be fermented by microorganism such as *Saccharomyces cerevisiae* or also known as baker's yeast to produce ethanol and carbon dioxide (Lin & Tanaka, 2006). *Saccharomyces cerevisiae* also can produce high amount of ethanol which gives concentration as high as 18% of the fermentation broth. In addition, *Saccharomyces* can survive on glucose and disaccharide sucrose and both sugars are simple sugars. Lin and Tanaka (2006) also said that sugars that in degradable form can reduced the amount of cost preparation since the yeast cells can metabolize the sugars directly. There are studies showing that there are several microorganisms can be used in fermentation such as *Saccharomyces cerevisiae*, *S. Diastaticus*, *Kluyveromyces marxianus*, *Pichia kudriavzevii*, *Escherichia coli* strain KO11, *Klebsiella oxytoca* strain P2 and *Zymomonas mobilis* (Zabed et al., 2014). *E. coli* is greatly used in fermentation because of its efficiency converting sugar into ethanol, able to floc during growth and high tolerance to ethanol (Zabed et al., 2014). *Z. mobilis* actually produce more ethanol (97%) than *S. cerevisiae* (90-93%). *S. cerevisiae* catalyze sugar by Embden-Meyerhof (EM) pathway also known as glycolysis while *Z. mobilis* catalyze glucose by Entner-Doudoroff (ED) pathway and the only microorganism that are able to metabolize glucose anaerobically (Lin & Tanaka, 2006). Tortora et al. (2006) found that some bacteria can metabolize glucose without the glycolysis or the pentose phosphate pathway and the bacteria have the enzymes for the ED pathway. However, *Z. mobilis* only ferments glucose, fructose and sucrose making it hardly to replace *S. cerevisiae* (Zabed et al., 2014). According to Tortora et al. (2010), alcohol fermentation started when a molecule of glucose produced two molecules of pyruvic acid and two molecules of ATP. Then, two molecules of pyruvic acid converted into two molecules of acetaldehyde and two molecules of carbon dioxide. Lastly, two molecules of acetaldehyde reduced by two

molecules of NADH and catalyzed by alcohol dehydrogenase to form two molecules of ethanol (Tortora et al., 2010).

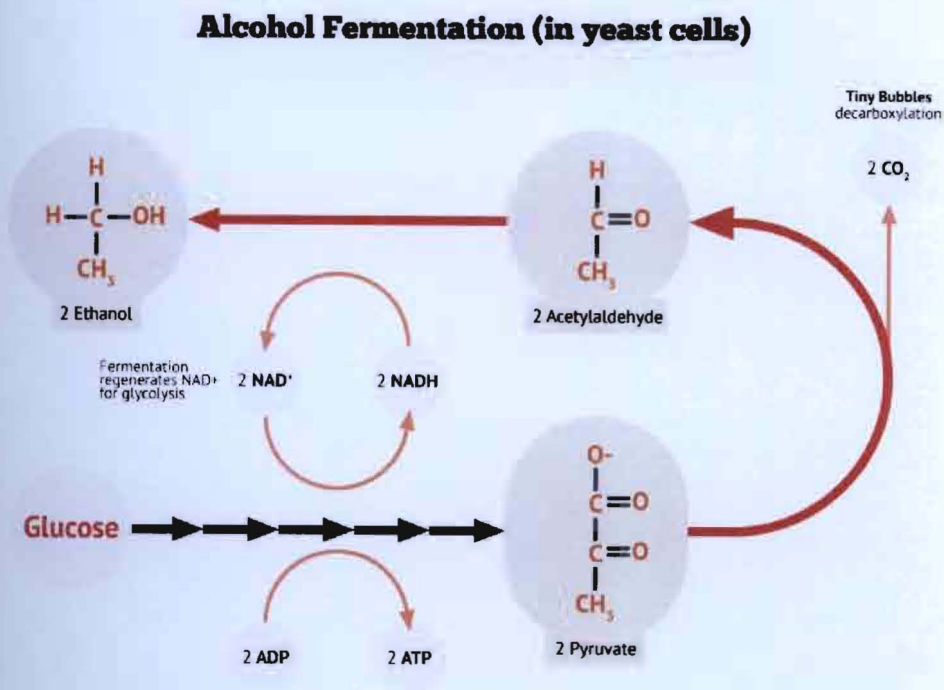


Figure 1: Alcoholic fermentation in the yeast cell

Source: <http://www.thechemicalblog.co.uk/what-are-the-chemical-reactions-involved-in-beer-making>

CHAPTER III

MATERIALS AND METHODS

3.1 Preparation of Sago 'Hampas'

The 'hampas' was put outside and was left for about 1 to 2 days. This was to remove any moisture contain inside the 'hampas'. Then, the 'hampas' was dried at under the sun for 24 to 72 hours and was ground to obtain 1 mm in size of substrate particles.



Figure 2: Drying of sago 'hampas'

3.2 Saccharification of Starch in Sago 'Hampas'

Seven percent (w/v) suspension of 'hampas' was be prepared in 0.1 M KH_2PO_4 (monopotassium phosphate) buffer solution at pH 4. The suspension was gelatinized at 85 to 90 °C for 15 minutes. Then, Liquozyme enzyme was added into the mixture. The suspension was stirred constantly for the next 20 minutes in order to ensure the mixture

mixed well. Next, the suspension was left at 60 °C and submerged in a water bath. Spirizyme was added and was left in the incubator shaker at 50 to 60 °C for 5 hours. The hydrolysate was filtered using 100 mesh sieve filter to separate it from residual lignocellulosic fiber and centrifuged at 12000 rpm for 15 minutes. The supernatant or also known as sago 'hampas' hydrolysate (SHH) was taken and analyzed for reducing sugars and glucose content. This is the end of the first cycle, this step was repeated until third cycle (Awg-Adeni et al., 2012).

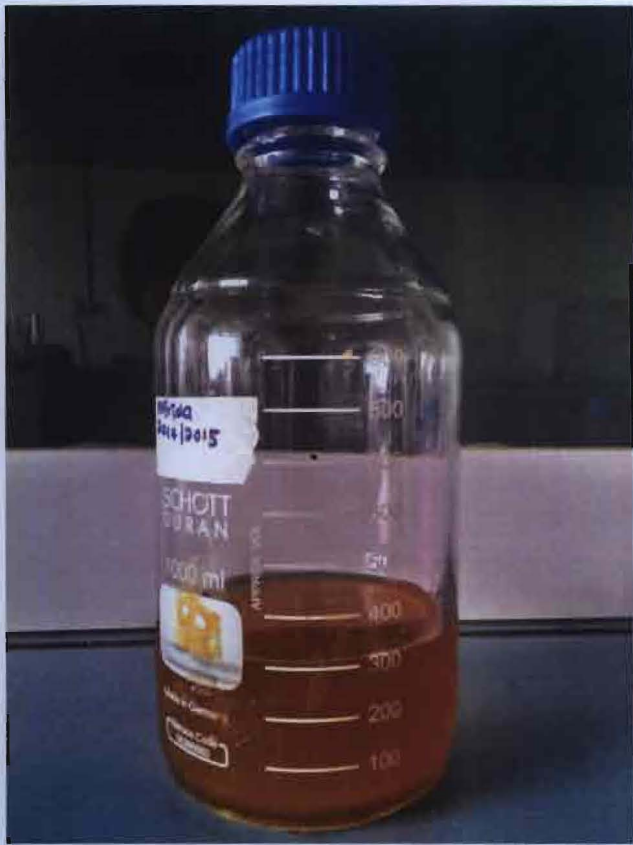


Figure 3: Sago 'hampas' hydrolysate

3.3 Preparation of Inoculum and Microorganism

The yeast or *Saccharomyces cerevisiae* (*S. cerevisiae*) were cultured on potato dextrose agar. The yeast then transferred into 100 mL inoculum media containing 20 g/L glucose